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14. ABSTRACT

We have previously shown that loss of TSC pathway components alters the timing of neuronal differentiation in the Drosophila eye and wing imaginal disc. To determine the mechanisms underlying this regulation of neuronal differentiation, we have 1) further defined the subtypes of photoreceptors that respond to loss of TSC, 2) tested in silico candidates for the regulation of differentiation in the eye, and 3) optimized conditions for an RNAi screen.Our preliminary data indicate that loss of TSC does not control neural differentiation through 5' TOP elements contained in the mRNA of proneural genes examined (CG11799, echinoid, moleskin, src). Consistent with this conclusion, our genetic analysis of the Drosophilahomolog of polypyrimidine tract binding protein, Hephaestus, indicates that loss of Hephaestus does not alter the timing of differentiation of photoreceptors in the eye. We conclude therefore that alternate mechanisms control this process. Our preliminary epistasisanalysis indicates that S6K is essential for the precocious differentiation seen in TSC clones, while loss of eIF4E does not affect the timing of differentiation.

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None provided.

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INTRODUCTION: Tuberous Sclerosis complex (TSC) is one of the most common forms of neurocutaneous disorders, affecting 1 in 6000 live births. This autosomal dominant disease typically presents in the pediatric age group with neuropsychiatric signs and symptoms of epilepsy, mental retardation and autism. It is poorly understood how loss of TSC leads to these neurological defects. We have shown that TSC plays a key role in controlling the timing of neuronal differentiation in *Drosophila* through the conserved insulin receptor (InR)/Tor kinase signalling pathway (Bateman &McNeill, 2004). The goal of our current research is to determine the mechanism by which TSC regulates the timing of neuronal differentiation. To uncover the pathway(s) downstream of TSC in the temporal control of neuronal differentiation we are taking three complementary approaches that will address both the transcriptional and translational outputs of the TSC pathway. We will 1) analyze *in silico* candidates of TSC translational control that we have identified using bioinformatics analysis, 2) conduct a screen of the *Drosophila* genome using double stranded RNAi to identify genes that mediate the temporal control of neural differentiation downstream of TSC and 3) analyze the Pointed P2 (PntP2) promoter. PntP2 protein is a primary target of RAS/mitogen activated protein kinase (MAPK) signalling that controls neuronal differentiation in the eye. Our preliminary data indicate that TSC transcriptionally regulates expression of the ETS domain transcription factor *PntP2*.

BODY: **Task 1. Analysis of candidates for TSC translational translational control identified in an *in silico* screen.** We have previously shown that loss of TSC in the *Drosophila* eye leads to precocious neural differentiation, without altering the specific cell fate decisions. Regulation of growth by the InR/Tor signaling pathway is mediated in part through translational control. S6 kinase is phosphorylated, and translation of 5'TOP-containing ribosomal transcripts is increased in response to this signaling. Could the timing of neuronal differentiation (a function of the Egfr pathway) depend on translation of 5'TOP-containing neuronal transcripts (whose translation depends on S6 kinase in the InR/Tor pathway)? To test this hypothesis, we conducted an *in silico* screen for messages that contain 5'TOPs and that may function in neuronal differentiation.

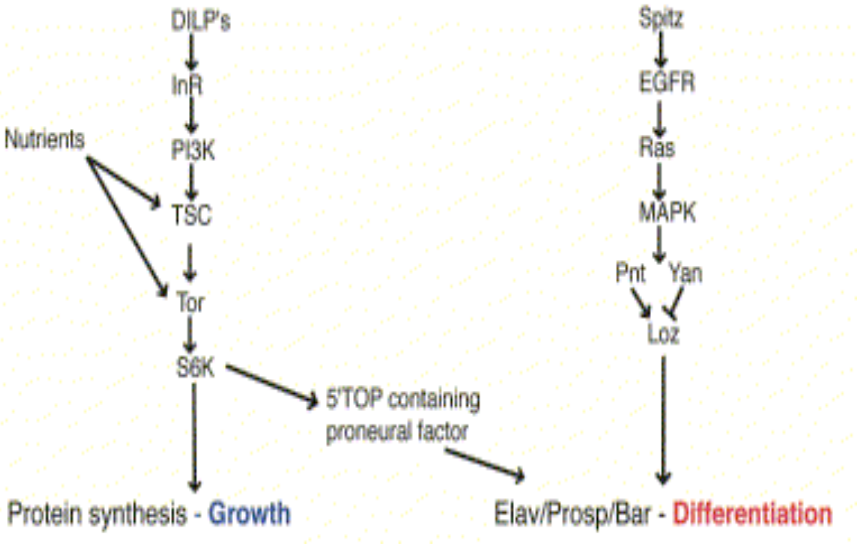


Figure 1. Model of growth and differentiation in the InR/TSC pathway via translational control of messages containing 5"TOPs.

5'TOP = 4-14 pyrimidine tract, start with a C, similar proportion of C/T, followed by GC rich sequence (not all of these behave as TOPs).
5'TOPs have been characterized in ribosomal proteins - and there are many ribosom proteins with 5'TOPs

Search Drosophila 5'UTR database for 5'TOP's:
Parameters: at least 4 pyrimidines at the 5' end of the transcript
Screened Flybase and Ensemble; 13608 annotated 5' UTRs.

Search of the *Drosophila* 5'UTR database for 5'TOPs revealed a number of mRNAs that contained 5'TOPs with roles in neuronal differentiation. Those highlighted in red were chosen for study because they had long 5'TOP sequences (msk, CG11799, Src64B, Src42A) or had other photoreceptor related functions (echinoid).

Epac	CTCTCC	MAPKKK cascade
Klg	CTTC	R7 differentiation
mbt	CTCT	serine/threonine kinase, photoreceptor cell development
Gap1	CTCT	Ras Gap
Rapgap1	CTCT	Ras GTPase activator activity
rg	CTTCTTTC	PKA anchoring, cone cell differentiation
argos	CTTC	EGF inhibitor
Hrs	CTCTT	HGF substrate.
Hep	CTTT	JUN kinase kinase
Gp150 CTTC		eye morphogenesis
ed	CTCT	echinoid , negative regulation of EGFR signaling
CG13030	CTTTT	R7 differentiation, RING finger domain,
msk	TTTTCTTCTTCTTCT	MAPK regulation
CG11799	CTTCTTCTTCTCCTTC	Forkhead TF factor
Src64B	CTCTCTCTTTC	Kinase, expressed posterior to furrow
Src42A	CTTTT	Oncogene, kinase

These data suggested the hypothesis that the transcript levels of these genes would be unaffected by TSC signaling, yet protein levels would be increased upon loss of TSC. To test this hypothesis, therefore we first needed to obtain or generate specific antibodies to candidate genes, and examine their expression in TSC loss of function clones.

Mnf (CG7719)

To examine if Mnf protein levels vary in TSC clones, we initially generated a peptide antibody against Mnf. and tested it on western blots and through immunofluorescence (IF). These antibodies recognize Mnf on western blots, but but did not work well for IF (data not shown). To obtain an antibody to examine Mnf in TSC clones in the eye imaginal disc, we then generated N-terminally GST-tagged Mnf constructs. Two rats were injected with the GST-tagged protein. Both generated antibodies that recognized Mnf on western blot and in the eye imaginal disc (data not shown). Both antibodies were then used to examine *tsc* and *pten* clones.

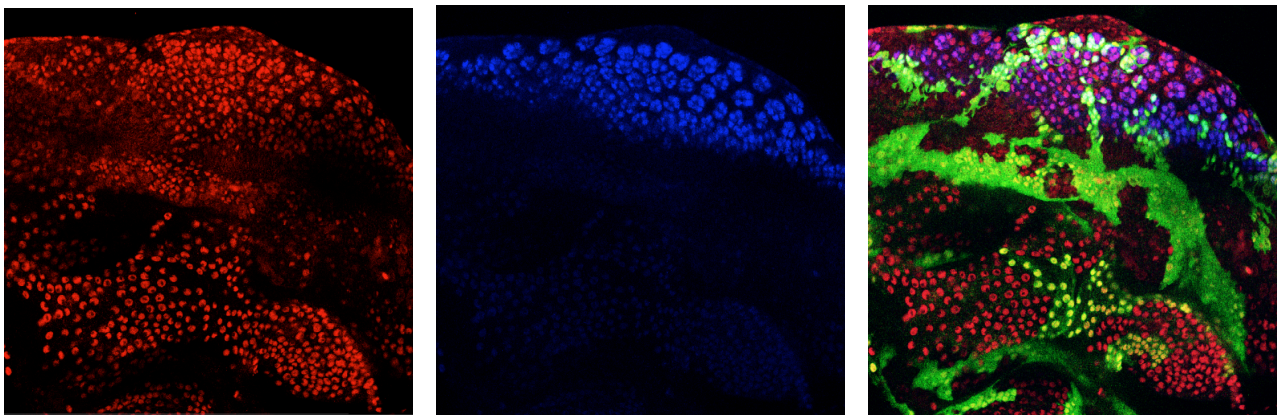


Figure 2. Mnf expression does not alter in *Tsc* clones. Clones of Tsc1-/- cells (marked by loss of GFP) did not alter expresson of Mnf (red).

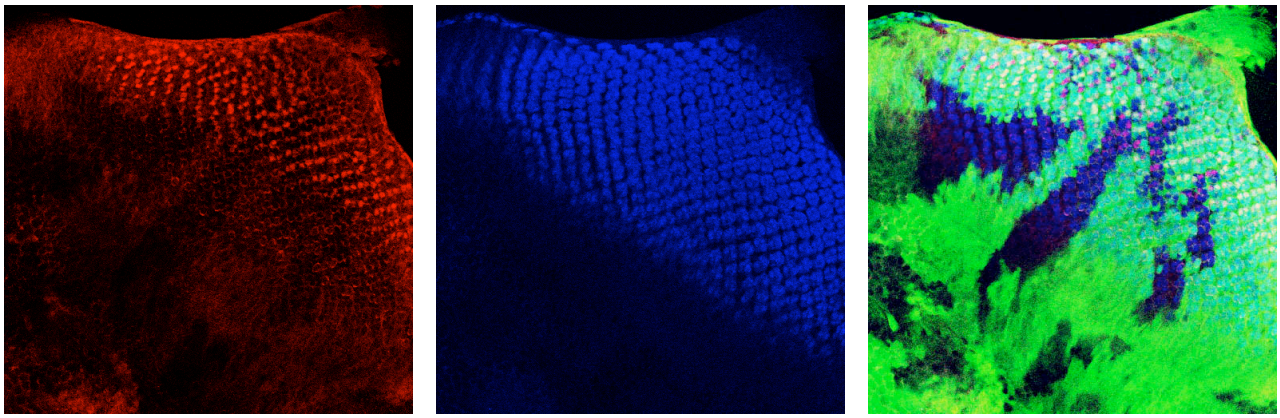


Figure 3. Overexpression of Mnf does not alter the timing of differentiation. Bar expression, nor Elav expression, changed in response to overexpression of Mnf (marked by expression of GFP).

Bar/Elav/GFP

Since loss of TSC or Pten does not alter Mnf expression (Figure 2), and overexpression of Mnf does not change the timing of differentiation (Figure 3) we conclude that TSC does not control differentiation via translational control of Mnf.

Src42A

Specific antibodies against Src42 and Src 64B were obtained as a gift from Dr. Jack Dickson (University of California, San Diego). These antibodies recognize Src42 and 64 in western blot analysis and in immunohistochemistry. Analysis of *Tsc1* clones revealed no alteration in the overall expression levels of Src42A (Figure 4) or Src64B (Figure 5).

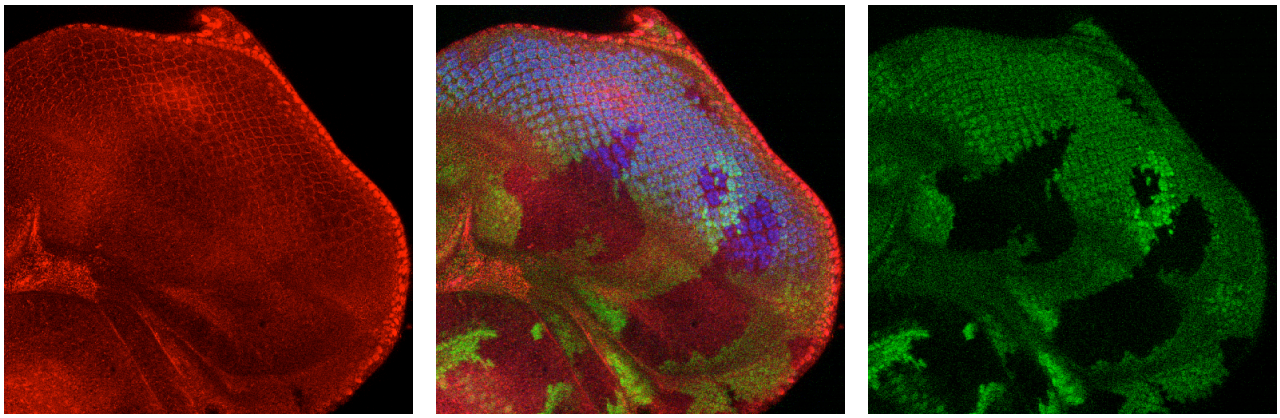
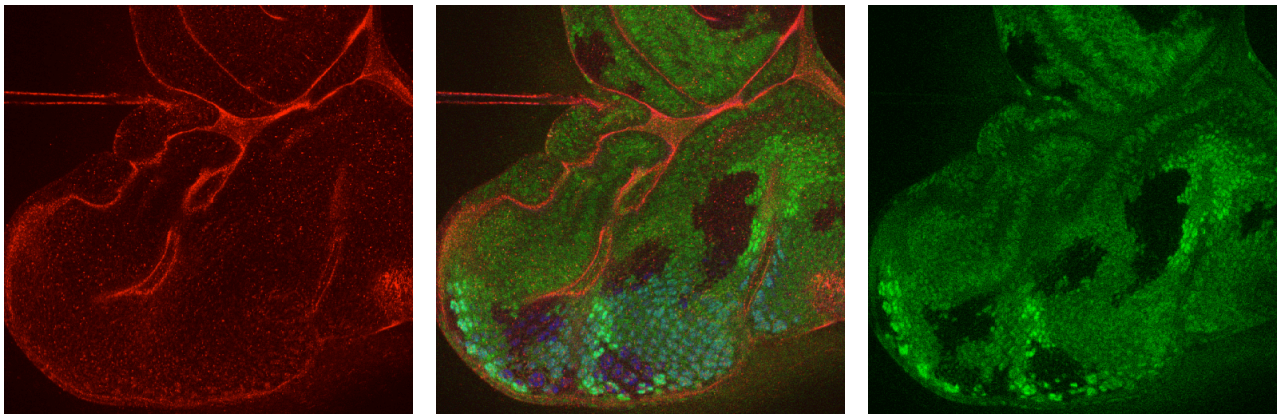


Figure 4. Src42A expression is not altered by TSC. Src42 protein levels(red) do not change in *tsc1* clones (marked by loss of GFP).

Src42A/Elav/GFP



Src64B/Elav/GFP

Figure 5. Src64B expression is not altered by loss of TSC. *tsc1* clones (marked by loss of GFP) were generated using the Flp FRT system. No significant alterations were detected with loss of tsc in clones.

Echinoid (Ed)

Echinoid (Ed) is a cell surface protein required to moderate Epidermal Growth Factor Receptor (Egfr) signaling during R8 photoreceptor selection in *Drosophila* eye development (Rawlins *et al.*, 2003). Although *ed* itself has only a short 5'TOP sequence, its function in the Egfr signaling pathway made it an interesting candidate for involvement in the timing of photoreceptor differentiation. Anti-Echinoid antibodies were a kind gift of Laura Nilson (McGill University, Montreal, Canada). No alterations in Echinoid levels were detect upon loss of TSC (Figure 6).

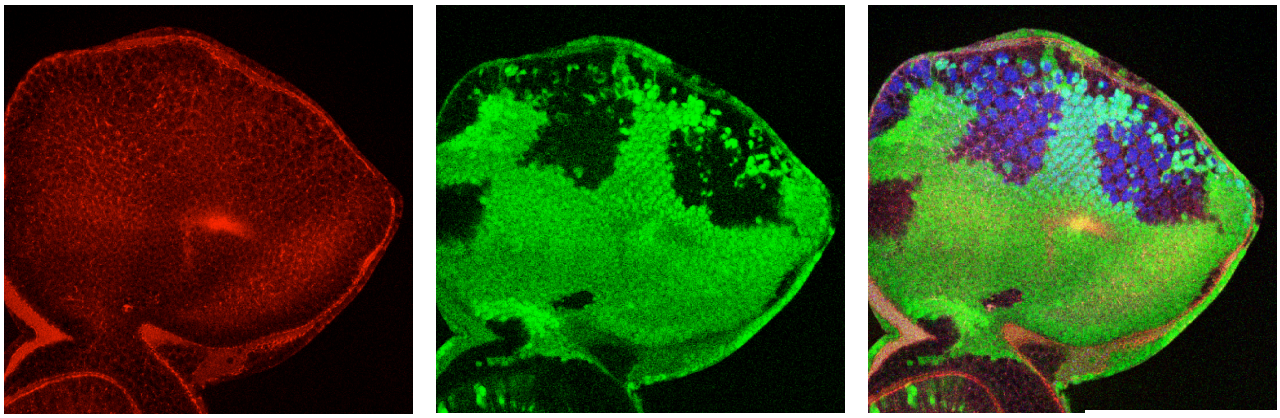


***Figure 6. Tsc1* clones do not change the expression of Echinoid**: tsc-/- clones were marked by loss of GFP (green). Echnoid expression (red) was unaltered. Elav (blue) marks photoreceptors.

Moleskin (Dim7)

The *Drosophila* homolog of vertebrate importin 7 (Dim7), Moleskin (Msk), has been found to bind pMAPK and is involved in its nuclear transport, with reported roles in embryonic and wing development (Lorenzen *et al.*, 2001). Msk is required for cell proliferation and survival in early eye development, as well as for correct ommatidial rotation (Vrailas *et al.*, 2006). Specific antibodies for Msk were a kind gift of Dr. Liz Perkins (Harvard University, Boston, USA). We generated clones of *msk* in the eye imaginal disc and found marked delays in the onset of neuronal differentiation, consistent with a proposed role of *msk* downstream of *tsc*. However we found that loss of TSC does not change the expression of Msk (Figure 7), suggesting that alterations of translation are not the underlying mechanism.

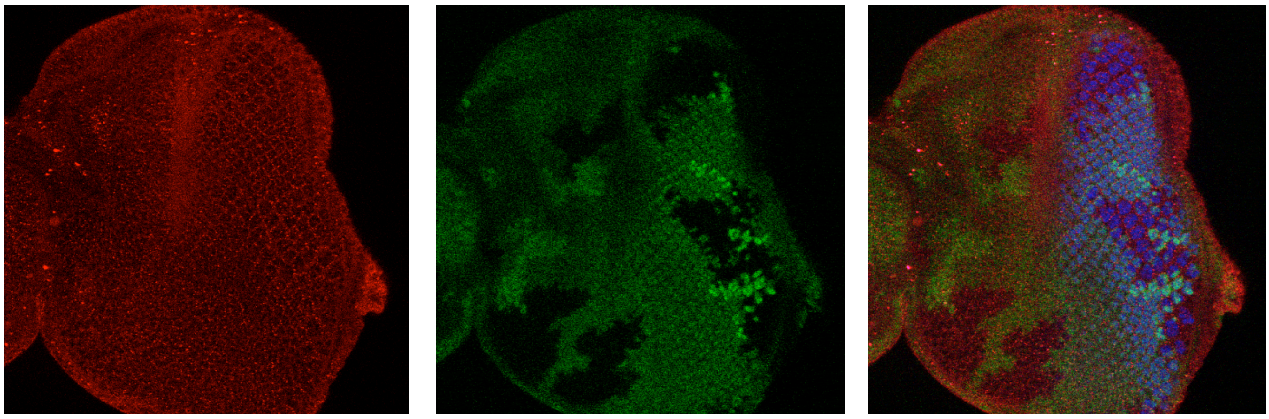


Figure 7. Dim7 does not show any changes in expression in *tsc1* mutant clones (marked by loss of GFP), which suggests it is not a part of the InR/Tor differentiation signaling pathway.

Dim7/Elav/GFP

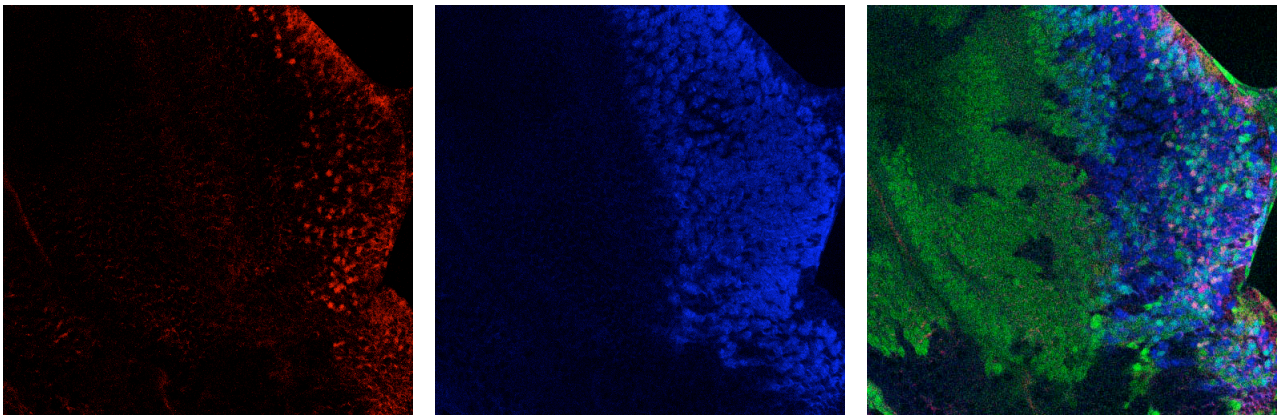
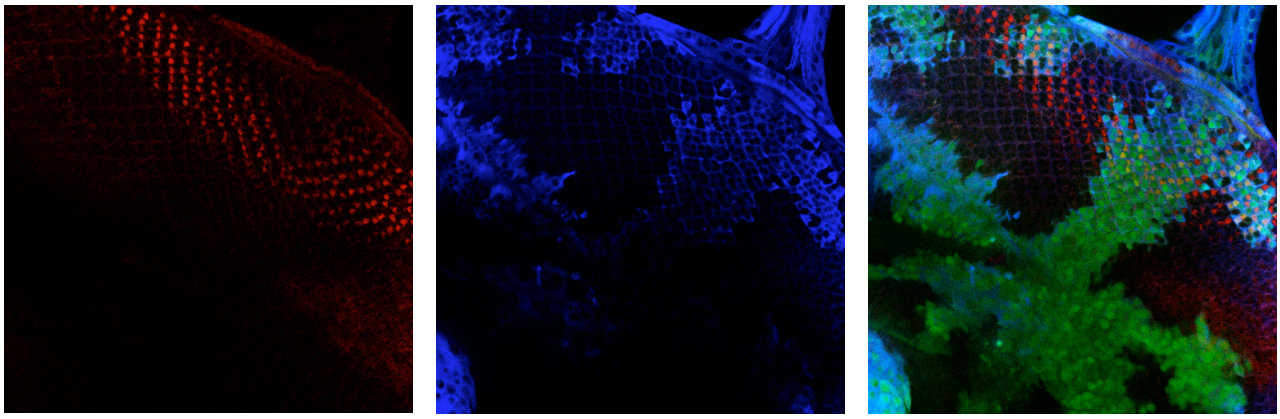


Figure 8. Loss of *msk* leads to delays in neuronal differentiation*Dim7* clones (marked by loss of GFP) alter the rotation of R1/R6 photoreceptors as marked by Bar (red) and distribution of ommatidia marked by Elav (blue).

Bar/Elav/GFP

Summary of Dim7: Although *dim7-/-* clones appear to have planar polarity effects at the *Drosophila* 3rd instar stage (Figure 8), no effects are seen in Dim7 expression in the absence of *tsc1* expression (Figure 7). There are also no changes in Bar expression when Dim7 is overexpressed (Figure 9). Since Dim7 has roles in embryonic development, as well as cell proliferation and survival in early eye development, the effects seen in *dim7-/-* clones may be due to defects in these aspects of development, rather than to a role of differentiation timing.

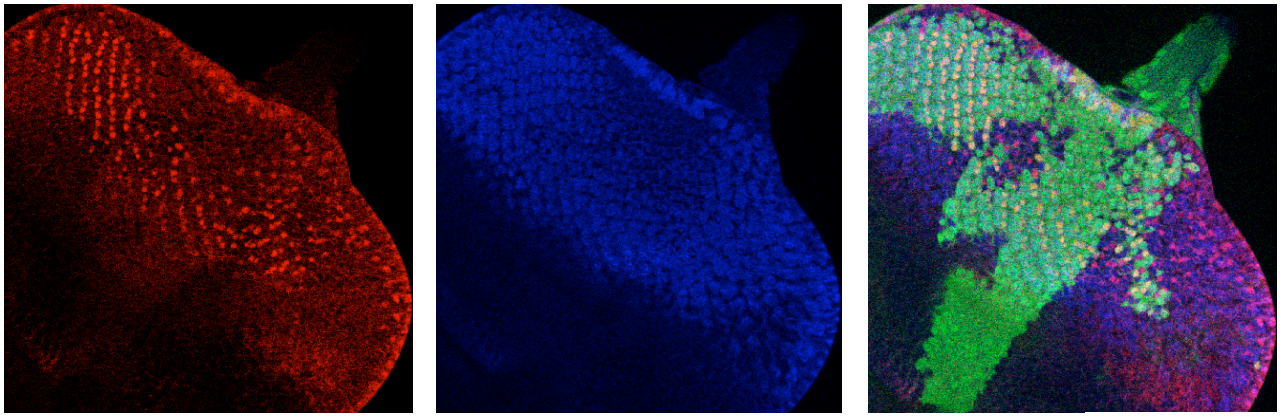


Bar/Dim7/GFP

Figure 9. Overexpressed msk does not alter the timing of differentiation. The onset of Bar expression is not altered in response to overexpression of Dim7 (marked by GFP).

Hephaestus

The *Drosophila hephaestus (heph)* gene encodes the homologue of mammalian polypyrimidine tract binding protein (PTB). PTB was first identified in vertebrates as a protein that binds to intronic polypyrimidine tracts (5'TOPs) preceding many 3' pre-mRNA splice sites (Garcia-Blanco et al., 1989). Many different functions have been identified for vertebrate PTB, including translational control and mRNA localization (Dansereau *et al*., 2002). To test the hypothesis that 5" TOPs are involved in the timing of differentiation downstream of *tsc*, we obtained *Heph* alleles and examined differentiation in the eye imaginal disc with antibodies to Bar and Elav. We found that *Heph* clones do not change timing of differentiation (Figure 10).



Bar/Elav/GFP

Figure 10. Loss of Heph does not alter the timing of differentiation in the eye disc. Although *heph-/-* clones (marked by the loss of GFP) show some disruption of Bar expression, there does not appear to be a change in differentiation timing within the clones.

Summary and future directions for analysis of Translational targets of TSC in neuronal differentiation

Mutant clones of TSC and PTEN, and overexpression clones of PI3K act to disrupt the timing of neuronal differentiation (Bateman and McNeill, 2004). These proteins are components of the InR/Tor pathway, a pathway known for its effects of growth, not neuronal differentiation. The InR/Tor pathway regulates growth by increasing the translation of 5'TOP-containing ribosomal transcripts. As such, it was hypothesized that the link between the InR/Tor pathway (containing TSC, PTEN, etc.) and the Egfr pathway (involved in differentiation) was 5'TOP transcripts with neuronal function. We proposed that increases in InR/Tor pathway signaling leads to increases in S6 kinase phohsphorylation and in turn translation of neuronal transcripts which would alter the timing of differentiation.

No neuronal proteins that are translated from transcripts with 5'TOPs (including Mnf, ed, dim7, Src42A and Src64B) showed any changes in levels of translation in the absence of *tsc* or *pten*, negative regulators of the InR/Tor pathway. Loss of Heph, a 5'TOP binding protein, did not alter the timing of differentiation. Our results suggest that 5'TOP-containing neuronal transcripts are not the link between the InR/Tor growth pathway and the Egfr differentiation pathway. Therefore we will continue our analysis of potential translational control by examining other translational mechanisms. Rather than an in silico approach, we will now identify transcripts associated with ribosomes under cases of increased InR signaling to identify TSC targets for neuronal differentiation.

Task 2) Screen for genes that function downstream of TSC in neuronal differentiation. Our preliminary data that loss of TSC leads to increased transcriptional regulation of Elav and PntP2 in the eye disc, and increased expression of the neuronal marker in S2 cells. We have been optimizing our RNAi protocol, as the initial tests of RNAi indicated that the 22C10 signal was not strong enough to be used in a high-throughput format with. We have been examining primary culture systems as an alternate system to S2 cells.

To better understand the signal transduction system downstream of TSC in neuronal differentiation, we have continued our genetic epistasis analysis of known components of the pathway in the eye imaginal disc. We have found that loss of eIF4E does not affect

the timing of differentiation, suggesting it is not a key element of the differentiation control. Strikingly, while loss of S6 has little effect on its own, double mutant clones of S6 and TSC leads to an abrogation of precocious differentiation, suggesting that S6 activation is crucial to the promotion of differentiation. Finally, we have also tested elements of the ecdysone pathway as potential downstream mediators of the TSC neuronal differentiation signal. Broad complex transcription factors have been shown to be regulated by the ecdysone pathway, which also regulates the timing of differentiation.

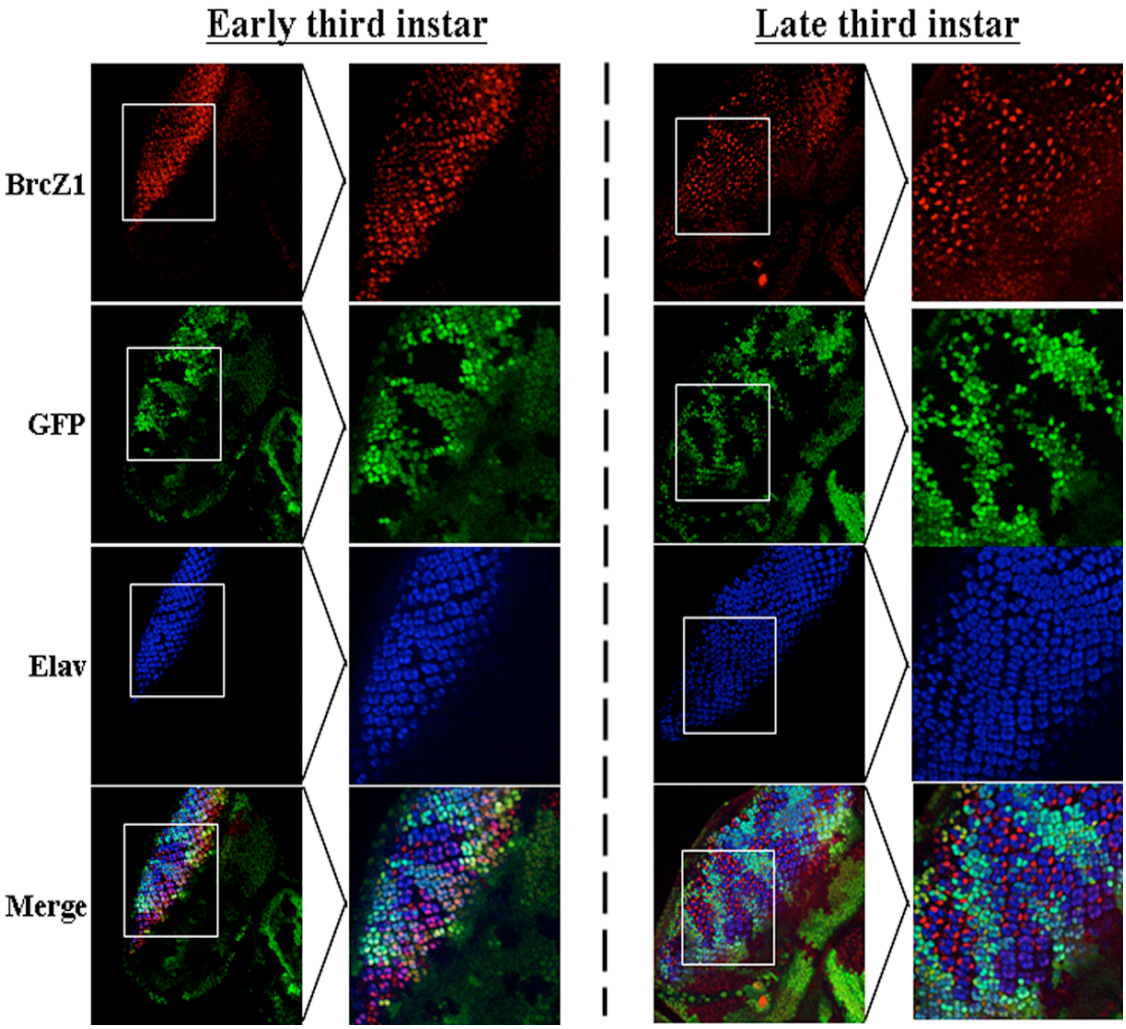


Figure 11. Loss of TSC leads to upregulation of Broad complex Z1. Broad Complex Z1 (BrcZ1, in red) is unaffected by loss of tsc1 (marked by loss of GFP). Left panel shows entire dics. Right panel shows magnification of area in white box.

We have found that loss of *tsc* also leads to upregulation of Broad complex expression in the eye disc (Figure 11). However the upregulation only occurs later in the differentiation,

during late third instar. Therefore regulation of Broad complex is unlikely to be primary factor in the regulation of the timing of neuronal differentiation.

Task 3) Analysis of PntP2. Our preliminary data indicated that loss of Tsc leads to increased expression of PntP2 transcript, as assessed by a PntP2 specific enhancer trap. We have verified that PntP2 expression increases with RT-PCR. To determine the enhancer elements responsible for the increase in PntP2 transcription upon loss of TSC, we have made enhancer-reporter constructs, and generated transgenic fly lines carrying the upstream elements of the PntP2 genomic region. Preliminary analysis of these lines indicates that a 4kb element can confer PntP2 expression in the eye imaginal disc. In parallel, we have determined that overexpressing PntP2 is not sufficient to drive precocious neuronal differentiation, suggesting other components downstream of TSC are needed for precocious differentiation. We are therefore also examining the enhancer region of the Elav promoter for TSC-responsive elements, in parallel with our studies on PntP2. These reporters will also be tested for usefulness in the RNAi screen (Task 2).

KEY RESEARCH ACCOMPLISHMENTS:

- 5'TOP containing genes were analyzed for roles downstream of TSC in the timing of neuronal differentiation in vivo. CG11799 (Mnf), Msk, Echinoid and Src were shown to not be altered in abundance in tsc loss of function analysis.
- PTB binding proteins were not effective in altering the timing of differentiation
- PntP2 enhancer analysis constructs have been generated and transgenic lines established.
- RNAi conditions are being optimized.
- Overexpression of PntP2 transcript was shown to be a consequence of loss of TSC in vivo.
- Gain of function and loss of function experiments demonstrated that PntP2 is necessary but not sufficient to mediated precocious neuronal differentiation downstream of TSC.
- Loss of S6K can block the precocious differentiation seen in *tsc* -/- clones, indicating an important role for S6K in the neural differentiation seen upon loss of TSC.
- Broad Complex Genes are upregulated by loss of TSC in vivo.

REPORTABLE OUTCOMES. A manuscript is currently being written to report the increase in PntP2 transcription in TSC mutant clones, and the necessary but not sufficient effects of PntP2 expression (Bateman et al, 2007) . A peer-reviewed literature analysis has been written on the intersection of Insulin Receptor signaling and neuronal differentiation (Bateman & McNeill, 2006)

CONCLUSION: Our studies have identified two new downstream targets of the TSC pathway in photoreceptor differentiation in the *Drosophila* eye; PntP2 and Broad Complex. We have tested the hypothesis that 5' TOP containing transcripts and found no evidence to support this model, therefore other modes of translational control will be assessed. We have determined that while PntP2 expression is necessary for neuronal differentiation, and its expression is altered by TSC activity, overexpression of PntP2 is not sufficient to account for the effects of TSC on neuronal differentiation. Future studies will focus on identifying additional targets and assessing if they are necessary and sufficient for the neuronal differentiation phenotypes observed upon loss of TSC.

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Cellular and Molecular Life Sciences

Visions & Reflections

Insulin/IGF signalling in neurogenesis

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Introduction

A fundamental challenge during the development of any complex organism is the coordination of proliferation and differentiation. In the case of neurogenesis, cells must exit the cell cycle and undergo a complex programme of gene expression and morphological changes. This requires the action of multiple secreted ligands which, by binding to their target receptors on the cell surface, control the course of neuronal cell fate in a spatiotemporal manner. Neurogenic organs are wholly dependent on prior proliferation to provide enough cells to generate the mature tissue. There are often assumed to be two sets of independent signalling pathways: one which controls proliferation and a second which controls differentiation. In this context, neuronal differentiation might be seen as a default pathway that occurs as a result of growth factor removal. Surprisingly, however, the same pathway often regulates both proliferation *and* differentiation. In this review we discuss the role of the insulin receptor (IR) and the type I insulin-like growth factor receptor (IGF-IR) receptor tyrosine kinases (RTKs) in neuronal differentiation by comparing knowledge about vertebrates with insight gained from studies in *Drosophila*. Evidence from vertebrates and flies suggests that, in certain developmental contexts and cell types, IR/IGF-IR signalling plays an important role in the differentiation of neurons.

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Insulin/IGF signalling in vertebrate neurogenesis

Although the role of IR and IGF-IR signalling in cell proliferation has been clearly demonstrated, the potential role of this group of RTKs in neuronal differentiation has received less attention. Insulin is best known for its role in glucose uptake and metabolism, whereas the insulin-like growth factors (IGFs) are well characterised as growth-promoting peptides [1]. Expression studies of the IR and IGF-IR have demonstrated that both of these RTKs are expressed in the nervous system [2, 3], suggesting that they function in neuronal development. The IR is widely expressed throughout the adult brain and concentrated expression is found in the hypothalamus, olfactory bulb and pituitary [3–5]. In addition, the IGF-IR is expressed in many embryonic tissues but high levels of expression are seen in the developing cerebellum, midbrain, olfactory bulb and the ventral floorplate of the hindbrain [2].
In cultured cells, insulin and IGF-I do not always act as mitogens. For example, in mouse fibroblast cell lines, insulin and IGF-I are very poor mitogens [6]. Insulin and IGF-I can also activate neurogenesis in ex vivo and cultured cell lines [6–11]. H19-7 rat hippocampal cells proliferate at 34 °C in response to serum and differentiate to a neuronal phenotype at 39 °C when treated with basic fibroblast growth factor (bFGF). However, expression of the IGF-IR allows HC19-7 cells to differentiate at 39 °C in response to IGF-I independent of bFGF [9]. In E14 mouse striatal primary neural stem cells (NSCs), the action of insulin/IGF-I to activate either proliferation

or differentiation is dependent on the passage number of the cells. NSCs isolated from neurospheres after two rounds of culture for 1 week differentiate to a neuronal phenotype in response to treatment with IGF-I [7]. Interestingly, the neurogenic action of IGF-I could be potentiated by the addition of brain-derived neurotrophic factor (BDNF), suggesting that these factors can act synergistically to promote differentiation. Conversely, treatment of similar NSCs from primary cultures with IGF-I caused individual cells to proliferate rapidly rather than differentiate [8]. Therefore, the ability of insulin/IGF to promote either differentiation or proliferation depends on the cell type and conditions.

What do the phenotypes of *Ir* and *Igf1r* mutant animals tell us about the role of these RTKs in neurogenesis? *Ir–/–* null mice develop normally but die shortly after birth due to severe diabetic ketoacidosis [12], suggesting that the IR is not required for neuronal development. Moreover, a neuron-specific disruption of the *Ir* gene in mice did not affect brain development or neuronal survival [13]. In contrast, *Igf1r–/–* mice have reduced brain size and altered brain structures, including a marked increase in the density of neural cells in the spinal cord and brainstem [14]. Furthermore, detailed examination of cochlear development has shown that development of this sensory organ is severely impaired in *Igf1r–/–* mice [15]. A significant decrease in the number of auditory neurons along with aberrant expression of early neural markers suggests that neuronal differentiation in the inner ear is delayed in these mice. Recent studies have also shown that IGF-I is required for differentiation of neuroblasts in the otic vesicle in chick [16]. Moreover, differentiation of neurons derived from mouse olfactory bulb stem cells requires IGF-I [17]. Thus, in mice the IGF-IR seems to be essential for correct central nervous system (CNS) development, while the IR may either be redundant or play a more subtle role.

What are the intracellular signalling cascades by which the IR and IGF-IR RTKs have the potential to control differentiation? In mammalian systems, insulin stimulation has been shown to cause activation of the Ras/mitogen-activated protein kinase (MAPK) pathway [18–20]. Activation of MAPK by the IR is independent of the role of this receptor in glucose homeostasis since inhibition of MAPK activation does not affect the metabolic actions of insulin [21]. Ligand binding to the IR results in tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins and/or Shc, which, through the adaptor protein Grb2, results in recruitment to the membrane of SOS for the activation of Ras (Fig. 1) [22, 23]. MAPK activation is the most well defined route by which IR/IGF-IR signalling might control neurogenesis during development. The first *in vivo* evidence for insulin stimulation of Ras came from the demonstration that insulin-induced *Xenopus* oocyte maturation is blocked by an antibody which inhibits Ras [24]. More recently, knock-out mice studies have shown that MAPK activation by insulin *in vivo* is dependent on IRS-1 [25]. In cultured cells, activation of MAPK is required for nerve growth factor (NGF)/epidermal growth factor (EGF)-dependent differentiation of PC12 cells [26]. Activation of MAPK in PC12 cells causes phosphorylation of target transcription factors and consequent reprogramming of gene expression to a neuronal fate [27]. Activation of MAPK by an IR/IGF-IR receptor-dependent mechanism has the potential to activate a similar neurogenic switch in target cells in the developing nervous system.

The other pathway which is activated by insulin/IGF receptor stimulation is PI3K/TOR signalling (Fig. 1). PI3K/TOR kinase signalling is known to regulate growth through the control of ribosome biogenesis and protein synthesis [28]. PI3K catalyses the conversion of PIP2 to PIP3, a process which is reversed by the lipid phosphatase PTEN. Growth control is mediated through TOR by the activation of S6K and the translation initiation factor eIF4E. The possible role of PTEN in the nervous system has been studied by several groups using conditional knock-out strategies. Although PTEN is not essential for cell fate determination in the CNS overall [29, 30], a dramatic effect was observed in glial cells. Yue et al. [31] used GFAP-cre to generate *pten–/–* cells in the CNS and observed premature differentiation of Bergmann glia in the early postnatal brain. The premature differentiation of *pten–/–* glia resulted in layering defects and subsequent aberrant migration of granule neurons. These data support a role for PTEN acting as a positive regulator of differentiation in certain cell types in the brain.

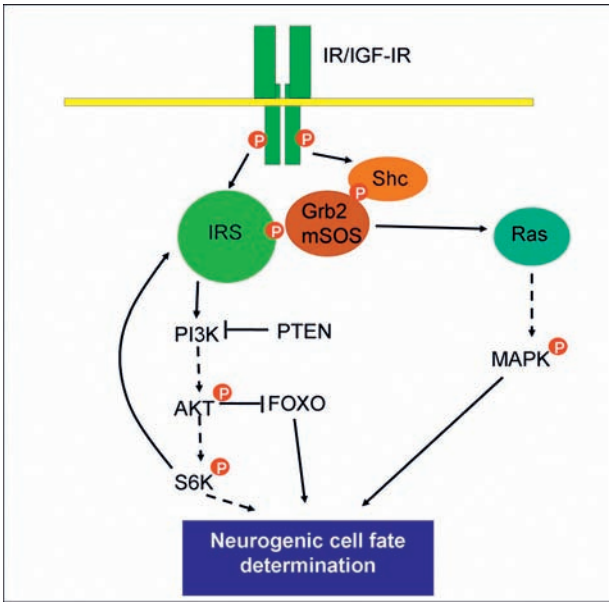

Figure 1. Potential pathways by which insulin/IGF signalling can regulate neurogenesis.

Insulin receptor signalling in *Drosophila*

Unlike vertebrates, *Drosophila* has a single RTK of the insulin receptor family (DInr). Expression of the DInr is ubiquitous during early stages of embryogenesis, but becomes enriched in the developing nervous system [32, 33]. The DInr can be activated by one of seven *Drosophila* insulin-like peptides (DILPS). Three of the DILPS are produced by seven neurosecretory cells within the brain. Flies in which these neurosecretory cells have been ablated are phenotypically similar to *dInr* mutants and have some features that are analogous to diabetes [34]. The DInr is required for growth during development and to attain full adult size [35]. Hypomorphic *dInr* mutants are developmentally delayed and have reduced size due to decreased cell number and cell size [36], suggesting that the role of the DInr during development is analogous to the IGF-IR. *dInr–/–* animals have defects in the development of embryonic central and peripheral nervous systems [32]. Unfortunately, this phenotype has not been studied in detail and so it is not clear whether embryonic neurons in *dInr* mutants are lost due to an inhibition of neurogenesis, proliferation, or indirectly though neuroblast apoptosis. In the developing eye, photoreceptor neurons do not absolutely require the DInr for neurogenesis; however, in the absence of the DInr, neuronal differentiation is significantly delayed [37]. Unlike activation of Ras/MAPK signalling, which is able to induce ectopic neurogenesis in the eye field, activation of DInr signalling modulates the timing of the differentiation programme. These findings suggest that the role of DInr signalling in neuronal differentiation is to act synergistically with other neurogenic pathways, such as EGF receptor (EGFR) signalling.

Does the DInr regulate the same intracellular signal transduction pathways as its mammalian counterparts? In *Drosophila* tissue culture cells, stimulation with mammalian insulin causes rapid phosphorylation of MAPK [38–40]; however, to date this has not been reported *in vivo*. Over-activation of MAPK signalling in the developing eye in *Drosophila* causes the formation of ectopic photoreceptor neurons [41, 42]. Over-expression of the DInr in the eye causes over-proliferation and, although the normal complement of photoreceptors are produced, there is a disruption in the patterning of the eye [36]. Interestingly, the patterning defect caused by over-expression of the DInr is similar to the planar cell polarity defects seen with mutations in EGFR signalling [43, 44], suggesting there may be cross-talk between these two pathways *in vivo*.

Chico, the *Drosophila* IRS, contains conserved putative binding sites for Drk, the homologue of the adaptor protein Grb2 [45]. Oldham et al. [46] generated transgenic flies containing a version of *chico* in which the putative Drk-binding site had been mutated, and found that this mutant was able to fully rescue the growth defects of *chico–/–* flies. In contrast, if the binding site for the regulatory subunit of PI3K (p60) in Chico was mutated, there was a complete loss of function. Why then is the Drk-binding site in the *Drosophila* IRS conserved? It is possible that a low level of MAPK activation may contribute to the ability of the DInr to control proliferation, although this is unlikely since loss of *pten* was able to completely rescue the growth defects caused by loss of the *dInr* [46]. Alternatively, the DInr may only activate MAPK in certain developmental contexts, such as embryonic development. Interestingly, loss of one copy of the *dInr* gene was able to dominantly suppress the embryonic lethality caused by over-expression of Ras^{V12} [47].

Work in the last few years has shown that, as in vertebrates, activation of the *Drosophila* PI3K is dependent on DInr signalling [28]. Signalling downstream of PI3K via AKT (PKB), the tuberous sclerosis complex (TSC) and TOR kinase is also highly conserved in *Drosophila*. As in mammals, the DInr pathway regulates the growth of flies via S6K and eIF4E. Moreover, the timing of photoreceptor neurogenesis in the developing eye is controlled by the DInr through a PI3K-TOR-dependent mechanism [37]. How might DInr signalling control neuronal differentiation through PI3K-AKT-TOR signalling? One of the targets of AKT is the forkhead transcription factor FOXO. FOXO regulates the transcription of a diverse set of genes that are involved in processes such as control of cell proliferation and apoptosis [48]. In certain developmental contexts, FOXO may be able to regulate the transcription of neurogenic genes, thereby mediating a neurogenic response to DInr stimulation. Alternatively, PI3K/TOR signalling may inter-connect with the Ras/MAPK pathway. Recent studies in mammalian tissue culture cells and in *Drosophila* have demonstrated the existence of a positive feedback loop by which S6K is able to regulate IRS levels and phosphorylation [49]. This feedback loop gives PI3K-AKT-TOR signalling the potential to control MAPK activation (and potentially neurogenesis) by modulating the activity of the IRS.

Conclusions and future directions

Can we assimilate the studies from vertebrates and flies to gain a greater understanding of the role of insulin/IGF signalling in neurogenesis? In both systems, *Ir*/*Igf1r* null animals show defects in CNS development. Further studies are needed, however, to characterise these defects in detail. Such studies should help to correlate the known expression patterns of the IR and IGF-IR with the affected neuronal/glial cell types. The mechanism of action by which insulin/IGF signalling controls differentiation is most easily addressed in cell culture systems. Vertebrate cell culture studies suggest that insulin/IGF-stimulated differentiation may occur through activation of the Ras/

MAPK pathway. Analogous studies have not been performed in *Drosophila* cells although the increasing availability of *Drosophila* neuronal cell lines in combination with RNAi technology provides an excellent opportunity to identify novel neural targets of the DInr. Vertebrate whole-animal models also show that insulin activates the Ras/MAPK pathway. *In vivo* studies in *Drosophila* have yet to demonstrate that the DInr can activate the Ras/MAPK pathway; however, our recent data suggest that in the *Drosophila* eye, the DInr pathway can regulate Ras/MAPK signalling through a transcriptional mechanism that requires TOR [unpublished results]. In conclusion, there is good evidence from both vertebrates and flies to suggest that insulin/IGF signalling has a conserved role in both proliferation and neuronal differentiation. The choice between proliferation and neurogenesis depends on the particular cell type or developmental context.

The contribution of insulin/IGF signalling to neurogenesis may be context and/or cell type specific; however, the importance of fine spatiotemporal control of neuronal differentiation means that understanding the role of this pathway is of major importance. Small alterations in the wiring of the brain can have profound consequences on function, and there are abundant data to suggest that the cues for axonal guidance alter over developmental time. In addition, the competence of neural progenitors to produce neurons of different fates is altered over time during development [reviewed in ref. 50]. To generate a structure of such intricacy as the brain, growth and differentiation must be coordinated, and the insulin/IGF signalling pathway appears to have just such a function. The challenge for the future is to understand molecularly how proliferation and differentiation are coordinated by a single pathway.

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